Upsala J Med Sci 106: 189–195, 2001

Avian Antibodies Can Eliminate Interference Due To Complement Activation In ELISA

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ABSTRACT

Antibodies derived from egg yolk offer many advantages over mammalian antibodies in several aspects. Chicken antibodies do not activate the human complement system and are sometimes a more suitable choice in designing solid-phase immunometric assays than mammalian antibodies.

The material often recommended for immunological assays is serum. A freshly drawn serum sample contains an active complement system, which is inactivated during storage. Mammalian antibodies used in most immunological assays may activate the human complement system. Activated complement components will bind to the antibodies thereby partly block the antibody binding epitopes. We show that an active complement system in undiluted samples reduce the absorbance values by approximately 50 % when using goat antibodies but not when using chicken antibodies. This difference will cause erroneous test results that will vary depending on the handling of the samples. Chicken antibodies can be used to eliminate this interference problem.

INTRODUCTION

Immunological assays have gained widespread use for the determination of hormones and other serum proteins. Two-site immunoassays are frequently used in clinical assays due to their speed and sensitivity. However, there is a risk of interference of endogenous components in the test sample that will give rise to erroneous results (14). The problem of interference will increase as the sensitivity of the assay increases. Endogenous interferences are very difficult to predict and eliminate as they vary both between patients and in the same patient from time to time. The most well known disturbances are due to rheumatoid factors and human anti-mouse IgG antibodies (HAMA) (5). It has previously been shown that binding of complement may interfere in solid-phase immunometric assays utilizing monoclonal antibodies (4). Complement activation is less frequently discussed as a cause of erroneous results in clinical laboratories. This is a bit surprising as the effects of complement on immune complexes are well documented. Plastic surfaces (e.g. microtitre plates) activate the human complement system (10). When an antigen binds to a capture antibody, an immune complex is formed that may activate the complement system. The antibodies bound to a solid phase also appear to behave like immune complexes (e.g. cap-

ture antibodies in an immunological assay) and are capable of complement activation even in the absence of an antigen. Binding of C1q is the initial step in the classical pathway and the activation will result in covalent binding of C3b and C4b to the immune complex and formation of the C5b–9 complex (12, 13). The process will result in the deposition of several complement factors with molecular weights similar to IgG or higher. C4b is mainly bound to the Fab region of the antibody (2). Hence, it is not surprising if complement activation may block part of the antigen binding sites. There are also two complementary mechanisms that influence the size of immune complexes: prevention of immune precipitation and solubilization of preformed immune precipitates (9, 11, 15). The classical pathway of the complement system is involved in both processes.

Chicken antibodies do not activate the human complement system (7). We have therefore compared the effects of an active complement system on antigen binding utilizing goat and chicken antibodies. The purpose of the study was to investigate whether avian antibodies could be used to avoid interference by an active complement system and how different sample dilutions influence the assay.

MATERIALS AND METHODS

Serum sampling

Normal human serum (NHS) was obtained from healthy individuals. All blood donors gave informed consent prior to blood sampling. The blood was left to clot for 30 min and centrifuged for 10 min at 3000×g. The serum was removed and samples were aliquoted. Heat inactivated human serum (HIHS) was made for 30 min at 56°C. All samples were stored at –70°C until use.

Chicken assay

Microtitre plates (F96, Polysorp, Nunc-immuno plate, Nunc A/S, Roskilde, Denmark) were coated with 100 µL of affinity purified chicken anti-bovine serum albumin antibody (chicken α-BSA), 5 µg/mL (Immunsystem AB, Uppsala, Sweden) in PBS (Phosphate buffered saline, 0.75 M NaCl, 0.1 M NaH_2PO_4, 0.02% NaN_3, pH 7.2) overnight at 4°C. The plates were washed three times in NaCl-Tween (0.9% NaCl, 0.05 % Tween-20 and 0.02% NaN_3) between every step. One hundred microliters of chicken anti-insulin antibody (Immunsystem AB, Sweden), 0.1 mg/mL were used to block the remaining sites overnight at 4°C.

One hundred microliters of NHS or heat-inactivated NHS were added to the plates in duplicate and 1:2 dilution series in veronal buffer (0.15 M NaCl, 0.2 mM $C_8H_{11}N_2O_3Na$, 0.3 mM $C_8H_{12}N_2O_3$) were performed. The samples were incubated for 1 h at 37°C. After the washing step 100 µL biotin labeled BSA (Immunsystem AB, Sweden) 1:10 000 were added and incubated for 1 h at 37°C. The biotin labeled BSA was detected with alkaline phosphatase conjugated streptavidin and allowed to bind for 1 h at 37°C. Enzyme substrate S-104 (Sigma, St. Louis, MO, USA) was added and allowed to react for 20 min in darkness before the plates were read in an ELISA reader (SpectraMax 250) at 405 nm.

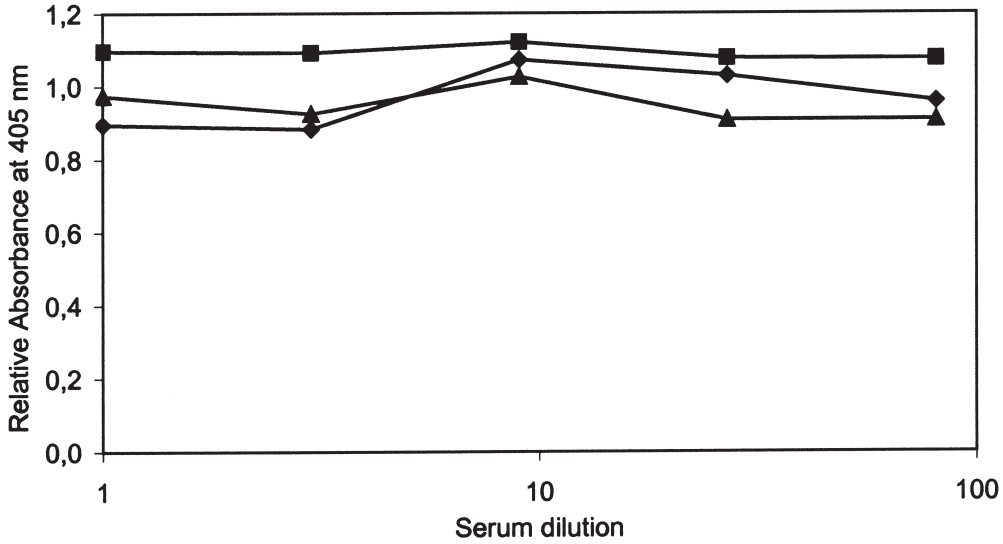

Fig. 1. ELISA results when chicken anti-BSA was used as capture antibody and addition of normal human serum (NHS). Results from three separate tests.

Goat assay

Plates coated with goat α-BSA antibody (The Binding Site, Birmingham, UK) were prepared as for the chicken assay except that blocking was done by 100 µL of 0.1 mg human IgG/mL (Kabi, Sweden).

RESULTS

Chicken anti-BSA as capture antibody

Preincubating the ELISA plates with NHS containing active complement did not inhibit the binding of biotinylated BSA to the chicken derived capture antibody (Fig. 1).

A similar binding pattern was obtained when the plates were incubated with NHS or HIHS (Fig. 2).

Goat anti-BSA as capture antibody

Preincubating the ELISA plates with undiluted NHS containing active complement inhibited the binding of biotinylated BSA to the capture antibody (Fig. 3). A reduction in BSA binding could be noticed when the plates were incubated with NHS in comparison with HIHS (Fig. 4). The difference was only present in dilutions less than 1:10.

DISCUSSION

Immunological assays, utilizing antibodies bound to a solid surface, are widely used for analysis of patient samples (e.g. sandwich and competitive immunoassays). The samples are often used undiluted or in low dilutions to increase sensitivity, reduce coefficients of variation and reduce assay time. Many hormones and cytokines are present in very low concentrations that do not allow dilutions of the samples prior to

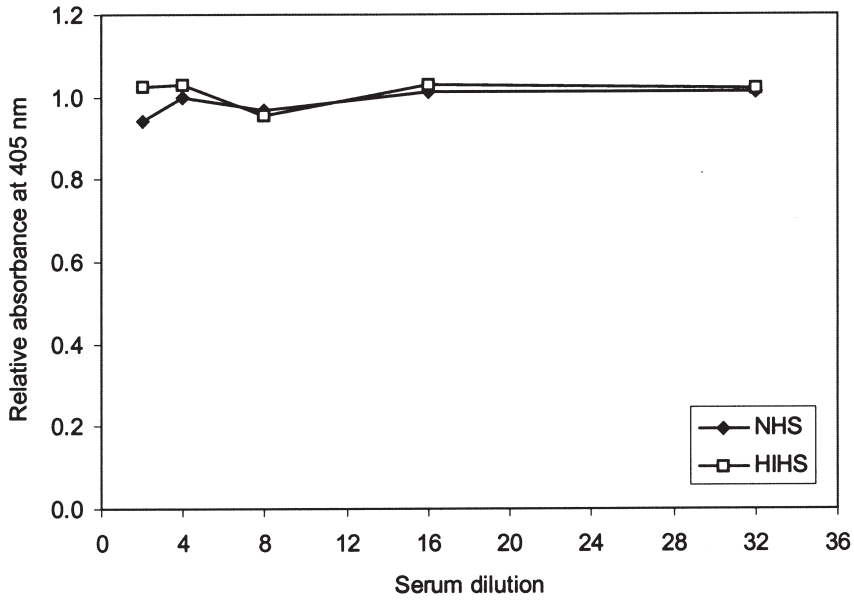

Fig. 2. ELISA results when chicken anti-BSA was used as a capture antibody and addition of normal human serum (NHS) or heat-inactivated human serum (HIHS).

analysis. Usually mammalian polyclonal or monoclonal antibodies are used as capture antibodies. Immune complexes containing mammalian antibodies and mammalian antibodies bound to a solid phase are known to activate the human complement system (1, 8).

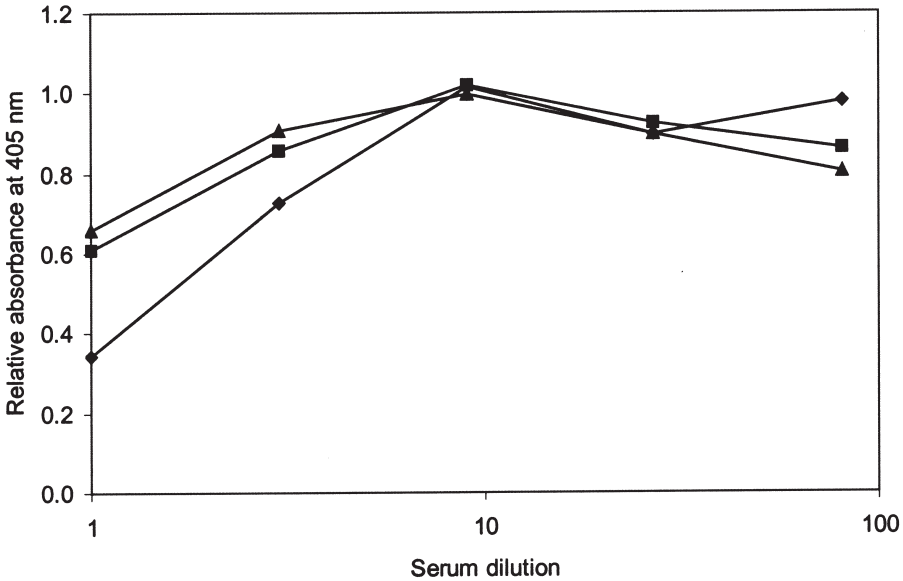

Fig. 3. ELISA results when goat anti-BSA was used as a capture antibody and addition of normal human serum (NHS). Results from three separate tests.

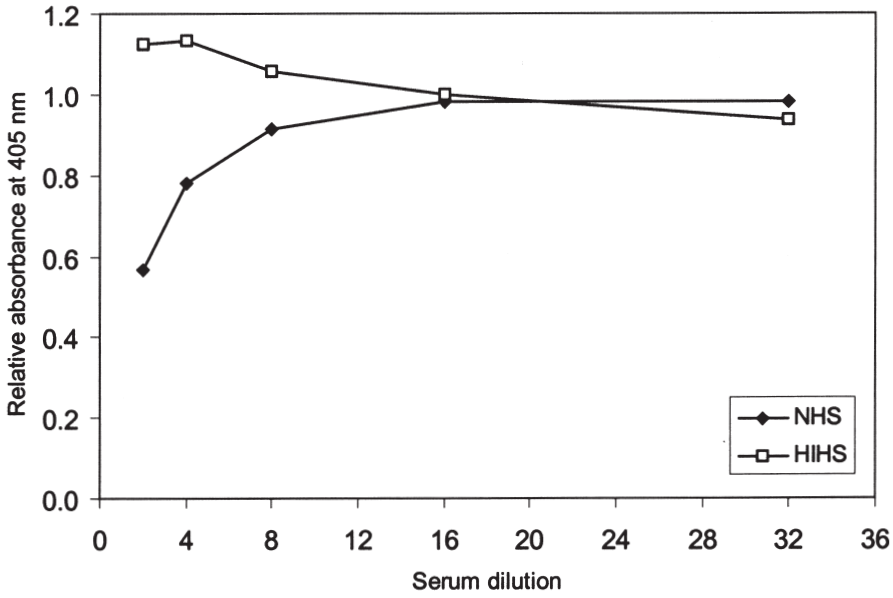

Fig. 4. ELISA results when goat anti-BSA was used as a capture antibody and addition of normal human serum (NHS) or heat-inactivated human serum (HIHS).

Activated complement component will bind to the capture antibody and may block the antigen-binding site (2). The complement deposition has been used in several studies for the evaluation of the complement function of patient samples (6, 16, 17).

Serum samples, and more recently, lithium-heparin plasma are the clinically most widely used samples. Immune complexes may activate the complement system in both these samples if the samples are analyzed within a few hours. If the samples are frozen at –20ºC or stored for a prolonged time, the complement system will be inactivated. The complement system is thus inactivated in controls and standards. The turnaround time in a clinical chemistry laboratory is usually well below two hours for short turnaround time (STAT) samples and point of care testing (POCT) (3). This means that STAT samples contain active complement while controls and standards do not. Method evaluations and reference materials are often performed on samples that have been frozen and the complement system is inactivated. The effects of complement activation are therefore usually not investigated. If complement activation interferes with the assay this will result in erroneous results when analyzing STAT samples.

We have compared goat and chicken antibodies as capture antibodies in ELISA. Goat antibodies are often used in commercial assays and are known to activate the human complement system. We found that complement activation blocked up to approximately 50% of the antigen binding when using goat antibodies as capture antibodies. This may result in a change in result by a factor 2 or more depending on the slope of the standard curve. The complement inhibition was observed in undiluted and samples that were diluted less than 1:10. In higher dilution the effect disap-

peared. The activated complement components in undiluted samples blocked the binding of the antigen to the mammalian goat antibody. It has previously been shown that sera from different individuals have varying complement-fixating capability (6, 16) which is reflected by the different degrees of inhibition noted with the goat antibody and high concentrations of NHS. An active complement system in the samples had no effect on the antigen binding when chicken antibodies were used as capture antibodies. Plastic surfaces are known to activate the complement system. The difference between goat and chicken antibodies indicates that the complement activation is not due to the plastic surface but to the antibodies used in the assay.

Chicken antibodies also offer other advantages to mammalian antibodies. Due to the evolutionary difference chicken IgY will react with more epitopes on a mammalian antigen, which will give an amplification of the signal. Chicken antibodies can also be used to avoid interferences due to rheumatoid factors, human anti-mouse IgG antibodies (HAMA) or Fc-receptors. A laying hen produces more yolk antibodies than a rabbit during the same time period. Therefore, chicken antibodies offer an interesting alternative to the traditional mammalian polyclonal antibodies.

Conclusion

It is important to consider complement activation when developing immunological assays that may be used for analysis of freshly drawn samples. The use of chicken antibodies may be one way of avoiding this interference problem.

ACKNOWLEDGEMENTS

Stiftelsen Samariten and NUTEK supported this work.

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